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Duplication of Hemolysin Genes in a Virulent Isolate of *Vibrio harveyi*

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***Vibrio harveyi* VIB 645, which is very pathogenic towards salmonids and produces extracellular product with a high titer of hemolytic activity towards fish erythrocytes, was found to contain two closely related hemolysin genes (designated *vhhA* and *vhhB*), whereas the majority of strains examined (11 of 13) carried only a single hemolysin gene. Both genes from VIB 645 were cloned and sequenced. The open reading frames (ORFs) of *vhhA* and *vhhB* shared a high level of identity (98.8%) and were predicted to encode identical polypeptides comprising 418 amino acid residues. The VHH protein shows homology to the lecithinase of *V. mimicus* and *V. cholerae*. Transformants of *Escherichia coli* containing the ORF of either *vhhA* or *vhhB* displayed weak hemolytic activity in rainbow trout blood agar. The hemolytic activity was very high when the ORF of *vhhB* was cloned in *E. coli* together with the native promoter. Surprisingly, the level of *vhh*-specific RNA transcript produced by VIB 645 was found to be very low. We conclude that the hemolytic phenotype of VIB 645 is not due to increased expression of one or both copies of the *vhh* gene.**

Vibrio harveyi is a gram-negative, luminous bacterium which is widely distributed in the marine environment (28, 29). Recently, this organism has emerged as a serious pathogen of marine animals. For example, the organism has been reported as a primary pathogen of cultured penaeid shrimp, especially in South America and Asia (1, 10, 18, 26, 33, 36). Additionally, *V. harveyi* has been associated with diseases in finfish (1, 8, 9, 15, 30) and pearl oysters (24). However, little is known about the pathogenicity mechanisms of *V. harveyi*. Liu et al. (19) considered that proteases, phospholipases, or hemolysins might be important for pathogenicity; cysteine protease has been reported as the major exotoxin to penaeid shrimp (16, 17, 20). Montero and Austin (21) suggested that the lipopolysaccharide might constitute the lethal toxin of *V. harveyi* E2 to penaeid shrimp. We have previously examined a large number of well-characterized *V. harveyi* cultures and found that the hemolysin activity in the extracellular product was involved in pathogenesis in salmonids. *V. harveyi* VIB 645, which was the most pathogenic isolate, produced extracellular product with the highest titer of hemolytic activity towards Atlantic salmon (1:256) and rainbow trout erythrocytes (1:32) (40).

In general, bacterial hemolysins have been suggested to be important factors of pathogenic vibrios by causing hemorrhagic septicemia and diarrhea in the host. Many of these hemolysins are well characterized, and genes encoding them have been cloned from *V. parahaemolyticus* (22, 35), *V. cholerae* (3, 27), *V. hollisae* (38), *V. mimicus* (12), *V. vulnificus* (38), and *V. anguillarum* (7).

In this study, we describe the cloning and characterization of the hemolysin gene of *V. harveyi* as a first step towards assessing the role of *V. harveyi* hemolysin in fish disease.

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MATERIALS AND METHODS

Bacterial strains and plasmids. Thirteen *V. harveyi* isolates from a diverse range of hosts and geographical locations and one type strain of *V. parahaemolyticus* were used in this study (Table 1). The cultures were obtained from the *Vibrio* collection in the Department of Biological Sciences, Heriot-Watt University, and were previously identified and confirmed for authenticity as *V. harveyi*, that is, cluster 1 of Pedersen et al. (25). The cultures were routinely grown on TNA (tryptone soy agar [Oxoid, Basingstoke, United Kingdom] supplemented with 1% [wt/vol] NaCl) at 28°C, overnight. *Escherichia coli* NM522 was routinely employed for bacterial transformations and for the maintenance and amplification of recombinant plasmids. *E. coli* TOP10F′ (Invitrogen, Groningen, The Netherlands) was used as a host for transformation with the pCR2.1-TOPO vector and its derivatives. LB medium (31) was used for the routine culturing of *E. coli* at 37°C. *E. coli* transformants were maintained on LB-Ap (LB supplemented with 100 μg of ampicillin per ml) agar. The plasmid pUC19 (37) was used as a vector for the cloning of restriction enzyme-digested DNA, and pCR2.1-TOPO (Invitrogen) was used for the cloning of *Taq* polymerase-amplified PCR products. Plasmids constructed in this study are listed in Table 2.

Isolation of DNA. Total DNA was prepared from *Vibrio* spp. by the procedure of Ausubel et al. (2). The rapid alkaline extraction method of Birnboim and Doly (5) was used for the preparation of plasmid DNA from *E. coli*.

TABLE 1. Strains of *V. harveyi* and *V. parahaemolyticus* used in this study

Strain	Name as received	Source	Country (yr) of isolation
VIB 571	*V. harveyi*	Sea bass	Spain (1990)
VIB 572	*V. harveyi*	Sea bream	Spain (1990)
VIB 645	*V. harveyi*	Sea bass	Tunisia (1993)
VIB 646	*V. harveyi*	Shark tank water	Denmark (1993)
VIB 647	*V. harveyi*	Sea bream	Greece (1992)
VIB 648	*V. harveyi*	Shark liver	Denmark
VIB 649	*V. harveyi*	Sea bream	Malta (1993)
VIB 651	*V. harveyi*	Shark tank water	Denmark (1994)
VIB 652	*V. harveyi*	Sea bass	Italy
VIB 653	*V. harveyi*	Sea bass	Turkey
VIB 658	*V. harveyi*	Sea bream	France (1990)
VIB 659	*V. harveyi*	Sea bass	Tunisia
VIB 661	*V. harveyi*	Sea bass	Tunisia (1992)
VIB 304	*V. parahaemolyticus*	LMG[a] 2850	

[a] LMG, culture collection of the Laboratorium voor Microbiologie, Universiteit Gent, Ghent, Belgium.

TABLE 2. Recombinant plasmids constructed in this study

Plasmid	Description[a]
pVHHH1	....pUC19 containing a 2.3-kbp *Hin*dIII fragment carrying the 5′ end of the *vhhA* gene (nucleotides 1 to 1017)
pVH2H	.....pUC19 derivative with 1.6 kbp *Hin*dIII fragment carrying the 3′ end of the *vhhA* gene (nucleotides 1065 to 1254)
p645H1-1	.....pCR2.1-TOPO containing a 1,257-bp PCR product carrying the ORF of *vhhA* (nucleotides −3 to 1254)
p645H1-15	...pCR2.1-TOPO containing a 1,257-bp PCR product carrying the ORF of *vhhB* (nucleotides −3 to 1254)
p645H2-1	.....pCR2.1-TOPO containing a 1,404-bp PCR product carrying the ORF of *vhhA* (nucleotides −61 to 1343)

[a] Nucleotide positions are relative to the first nucleotide of the translational start codon of the *vhh* gene.

Construction of a genomic DNA library. Total DNA from *V. harveyi* VIB 645 was completely digested using *Hin*dIII restriction endonuclease (MBI Fermentas, Sunderland, United Kingdom). The digested genomic DNA was mixed with *Hin*dIII-digested pUC19, which had also been treated with alkaline phosphatase (Roche Molecular Biologicals, Lewes, United Kingdom). This DNA mixture was ligated with T4 DNA ligase (Roche Molecular Biologicals) at 15°C overnight and used to transform *E. coli* NM522 by the method of Sambrook et al. (31).

Preparation of DIG-labeled DNA probes by PCR. Nucleic acid probes are described in Table 3, together with the primers that were used in their construction. The VPHP and VP2P probes are specific to the central portion and the 3′ end of the *V. parahaemolyticus tl* (thermolabile hemolysin) gene (35), respectively, and were prepared by PCR amplification from total DNA of *V. parahaemolyticus* VIB 304. The design of the primers was based on the nucleotide sequence of the *tl* gene published by Taniguchi et al. (35). All PCR primers were custom synthesized by MWG-Biotech AG (Ebersberg, Germany). The PCR was performed in a volume of 50 μl using 1 ng of template DNA, 5 μl of 10× buffer (supplied with the *Taq* polymerase), a 200 μM concentration of each deoxynucleoside triphosphate, a 0.5 μM concentration of each primer, 2 μM $MgCl_2$, and 2.5 U of *Taq* DNA polymerase (MBI Fermentas). The PCR conditions were 35 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C. A final extension of 7 min at 72°C was included. For the preparation of the digoxigenin (DIG)-labeled probes, 5 μl of 1 mM DIG-labeled dUTP (DIG-dUTP; Roche Molecular Biologicals) was included in the PCR. PCR products were resolved on a 1% (wt/vol) agarose gel, which was stained with ethidium bromide (1 μg/ml) before viewing on a UV transilluminator (UV Products, Prescott, Arizona).

Southern blotting and hybridization. DNA samples digested with restriction endonucleases were subjected to electrophoresis through a 1% (wt/vol) agarose gel and transferred onto a nylon membrane (Duralon-UV; Stratagene, Cambridge, United Kingdom) using a modified method originally described by Southern (34). Hybridization was performed overnight at 58°C in a solution of 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% (wt/vol) *N*-lauroylsarcosine, 0.02% (wt/vol) sodium dodecyl sulfate (SDS), and 2% (wt/vol) blocking reagent (Roche Molecular Biologicals). After hybridization, the membrane was washed twice for 5 min each at room temperature with low-stringency wash solution (2× SSC, 0.1% [wt/vol] SDS), and twice for 15 min each at 58°C with high-stringency wash solution (0.1× SSC, 0.1% [wt/vol] SDS). Detection of the DIG-labeled DNA was performed by an enzyme-linked immunoassay with an anti-DIG antibody conjugated to alkaline phosphatase (anti-DIG-AP; Roche Molecular Biologicals). The blots were developed at 37°C for 30 to 90 min using a chromogenic solution containing nitroblue tetrazolium (Sigma, Poole, United Kingdom) and 5-bromo-4-chloro-3-indolyl phosphate (*p*-toluidine salt; Sigma).

Colony blotting and hybridization. Transformed cells were plated onto LB-Ap agar. Bacterial colonies were transferred onto circular nylon membranes (Duralon-UV [8.2-cm diameter]; Stratagene) by the method of Sambrook et al. (31). Colony blots were hybridized and developed in the same way as Southern blots. Each positive bacterial colony from the master plate was subcultured and screened a second time. A single, well-isolated, positive colony was picked from the second screen and used for further analysis.

Cloning of PCR products of the *vhh* genes. PCR products were cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen) and as directed by the manufacturer. The design of PCR primers for the amplification of *V. harveyi vhh* genes was based on the sequences of the two partially cloned *vhh* gene fragments (VHHH1 and VH2H) from this study, and these primers are listed in Table 4. The template for the PCRs was *V. harveyi* VIB 645 DNA. The samples were subjected to 20 cycles of 1 min at 94°C, 30 s at 55°C, and 1 min at 72°C.

Nucleotide sequence analysis. Cloned DNA was sequenced by Cambridge BioScience (Cambridge, United Kingdom). Nucleotide sequences were translated using the ExPASy Translate tool (http://www.expasy.ch/tools/dna.html). Homology searches were performed using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple nucleotide or amino acid sequences were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/).

RNA extraction. *V. harveyi* was cultured on tryptone soy broth at 28°C overnight. Total RNA from 10^9 cells/ml was extracted using the RNeasy Mini Kit (Qiagen, Crawley, United Kingdom) according to the manufacturer's instructions.

Northern blotting and hybridization. RNA (2 μg) was separated by electrophoresis through a guanidine thiocyanate agarose gel (1.2% [wt/vol] agarose) with Tris-borate-EDTA running buffer and blotted onto a nylon membrane (Stratagene). Hybridization was performed at 58°C overnight in a solution composed of 0.25 M Na_2HPO_4, 1 mM EDTA, 20% (wt/vol) SDS, and 1.0% (wt/vol) blocking reagent (pH 7.2) (6). After hybridization, the blot was washed three times for 20 min each in prewarmed washing buffer (20 mM Na_2HPO_4; 1 mM EDTA; 1% SDS, pH 7.2). The DNA-RNA hybrids were detected by chemiluminescence (6).

Hemolytic activity assay. Fresh rainbow trout blood was collected by venipuncture from healthy animals maintained in a freshwater aquarium. The erythrocytes were washed three times with sterile phosphate-buffered saline (Oxoid). Blood agar plates were prepared by mixing 17 ml of melted LB agar (~55°C) and 3 ml of 10% (vol/vol in phosphate-buffered saline) washed rainbow trout erythrocytes in a 9-cm-diameter petri dish. *E. coli* clones were inoculated onto the blood agar plate, and hemolytic activity was determined by the appearance of a lytic zone on the surface of the blood agar following incubation overnight at 28°C.

Nucleotide sequence accession numbers. The nucleotide sequences reported in this work have been assigned GenBank accession numbers as follows: AF293430 for *vhhA* and AF293431 for *vhhB*.

RESULTS

Identification of hemolysin genes in *V. harveyi*. The VPHP probe (Table 3) was used to screen *Eco*RI digests of total DNA from the 13 *V. harveyi* strains listed in Table 1. At least one band was detected in all strains, indicating the presence of a hemolysin gene in *V. harveyi* that is very similar to the *tl* (thermolabile hemolysin) gene of *V. parahaemolyticus*. Two *Eco*RI fragments, with sizes of 8 and 15 kbp, were identified in VIB 645. Two *Eco*RI fragments, of 7 and 10 kbp, were also found in

TABLE 3. DIG-labeled DNA probes hybridizing to the *tl* gene of *V. parahaemolyticus*.

Probe	Primer	Sequences for PCR	Product size (bp)	Target for hybridization[a]
VPHP	Forward (VPF1)	5′-TGATCAGCACGCAAGAAAAC-3′	922	Nucleotides 104 to 1025
	Reverse (VPR1)	5′-GTTAGCGTCTCGAACAAGGC-3′		
VP2P	Forward (VP2F1)	5′-ATACTCACGCCTTGTTCG-3′	252	Nucleotides 998 to 1249
	Reverse (VP2R1)	5′-GGTACTCGGCTAAGTTGTTG-3′		

[a] Nucleotide positions are relative to the first nucleotide of the translational start codon of the *tl* gene of *V. parahaemolyticus* (GenBank accession no. M36437), which contains an ORF of 1,254 nucleotides (excluding the stop codon).

TABLE 4. Nucleotide sequences of PCR primers for amplification of the *vhh* gene of *V. harveyi*

Primer	Sequence (5′→3′)	Annealing site[a]	Product size (bp)
VHF1	ATCATGAATAAAACTATTACGTTACT	−3′→23	1,257
VHR1	GAAAGGATGGTTTGACAAT	1254→1236	
VHF2	GAGGACGTTTGGTGAGATAA	−61→−42	1,404
VHR2	ACGACGAATACAATCTCTGG	1343→1314	
VHF3	CTTATTAACGCCCAAAGTGC	−223→−204	1,677
VHR3	ATTGAGGTGCTCCAACAGAT	1454→1435	

[a] Nucleotide positions are relative to the first nucleotide of the translational start codon of the *vhh* gene of *V. harveyi*.

VIB 648. One band was observed in all other strains. Strains VIB 646, VIB 647, VIB 649, VIB 651, VIB 652, VIB 653, and VIB 658 produced a band at 8 kbp, whereas a 15-kbp band was detected in VIB 571, VIB 572, and VIB 659 and a 6-kbp band was detected in VIB 661 (Fig. 1). The VPHP probe was then used to examine total DNA from VIB 645 separately digested with eight further restriction enzymes (Fig. 2). In every case, two fragments were detected with the VPHP probe. This suggested that VIB 645 has two very similar, or possibly identical, copies of the hemolysin gene. The same is also likely to be true for VIB 648, although no further Southern hybridization analyses have been carried out on DNA from this strain. The two *Hin*dIII fragments detected in VIB 645 with the VPHP probe, with sizes of 1.5 and 2.3 kbp (lane 4 of Fig. 3), were selected for cloning in order to characterize the hemolysin genes.

Cloning of hemolysin genes from *V. harveyi*. A genomic DNA library of *V. harveyi* VIB 645, consisting of *Hin*dIII DNA fragments inserted into pUC19, was used to transform *E. coli* NM522. Transformed *E. coli* cells were screened by colony hybridization using the VPHP probe. Thirteen positive clones were isolated from an initial screen of approximately 12,000 *E. coli* transformants. Plasmid DNA was prepared from each clone and analyzed by *Hin*dIII restriction enzyme digestion. All thirteen clones contained an identical 2.3-kbp fragment. Further efforts to clone the 1.5-kbp fragment were unsuccessful. The recombinant plasmid containing the 2.3-kbp fragment was designated pVHHH1 (Table 2), and the inserted DNA was sequenced (data not shown). A partial open reading frame (ORF) was identified in the 2.3-kbp *Hin*dIII fragment and was predicted to encode a polypeptide with 87% identity to the N-terminal 341 amino acids of the *V. parahaemolyticus* thermolabile (TL) hemolysin.

Since the 2.3-kbp *Hin*dIII fragment lacked the 3′ end of the ORF of the hemolysin gene, a second probe VP2P (Table 3), which hybridizes to the 3′ end of the *V. parahaemolyticus tl* gene, was constructed for cloning the missing portion of the gene from VIB 645. Southern hybridization analysis of *Hin*dIII-digested DNA prepared from VIB 645 and probed with VP2P produced two fragments, of 1.7 and 3.8 kbp. Screening of the genomic library of VIB 645 yielded six positive clones. All six clones contained the 1.7-kbp *Hin*dIII fragment; the 3.8-kbp *Hin*dIII fragment was not obtained. The recombinant plasmid containing the 1.7-kbp *Hin*dIII fragment was designated pVH2H (Table 2), and the inserted DNA was sequenced (data not shown). A partial open reading frame, encoding a polypeptide with 70% identity to the C-terminal 54 amino acids of the TL hemolysin, was identified.

In order to clone a complete copy of each of the two hemolysin genes that were thought to be present in VIB 645, an alternative strategy based on PCR was developed. Three sets of PCR primers (Table 4) were designed for amplification of the hemolysin genes in VIB 645, based on the nucleotide sequences of the cloned DNA in pVHHH1 and pVH2H (Table 4). PCR products obtained from VIB 645 DNA, using VHF1 and VHR1 as primers, were cloned into pCR2.1-TOPO. Eighteen clones were isolated that contained the expected 1.3-kbp insert. Plasmid DNA was prepared from four different clones, and the inserted DNA in each of them was partially sequenced. When the nucleotide sequences were aligned, it became apparent that there were two types, with nucleotide differences at seven positions (195, 324, 393, 414, 441, 447, and 471, relative to the first nucleotide of the translational start codon). Two plasmids, p645H1-1 and p645H15-1, each containing an insert with a different sequence, were selected for complete sequencing of the hemolysin genes. The genes contained within p645H1-1 and p645H15-1 were named *vhhA* and *vhhB*, respectively.

The PCR products obtained using the primers VHF2 and

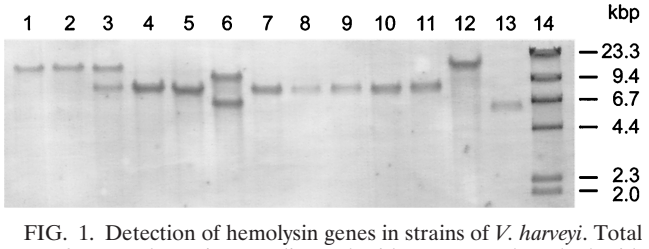

FIG. 1. Detection of hemolysin genes in strains of *V. harveyi*. Total DNA from each strain was digested with *Eco*RI and probed with VPHP. Lanes: 1, VIB 571; 2, VIB 572; 3, VIB 645; 4, VIB 646; 5, VIB 647; 6, VIB 648; 7, VIB 649; 8, VIB 651; 9, VIB 652; 10, VIB 653; 11, VIB 658; 12, VIB 659; 13, VIB 661; 14, DIG-labeled *Hin*dIII-digested λ DNA (molecular weight markers).

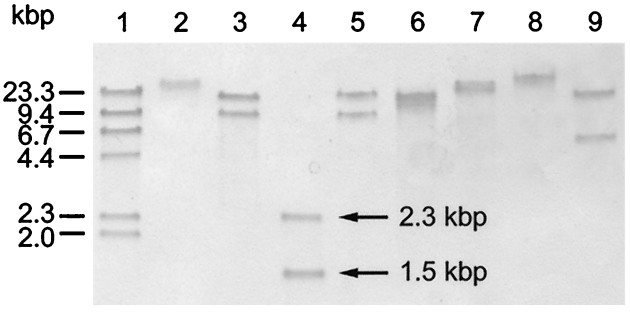

FIG. 2. Southern hybridisation analysis of restriction enzyme-digested DNA from *V. harveyi* VIB 645 probed with VPHP. Lanes: 1, DIG-labeled *Hin*dIII-digested λ DNA (molecular weight markers); 2, *Bam*HI; 3, *Eco*RI; 4, *Hin*dIII; 5, *Kpn*I; 6, *Pst*I; 7, *Sal*I; 8, *Sma*I; 9, *Xba*I.

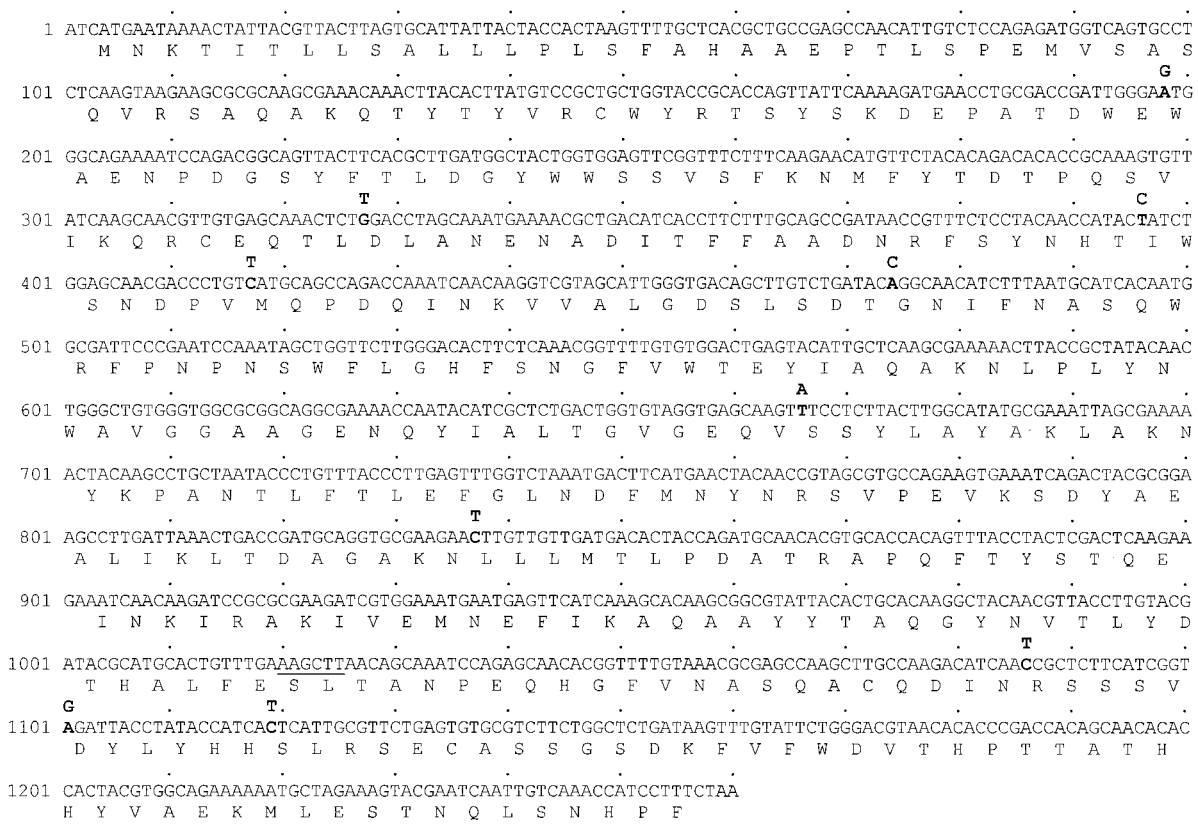

FIG. 3. Nucleotide sequence and translation of the *vhh* gene. Differences between the nucleotide sequences of *vhhA* and *vhhB* are indicated in bold face type; the lower nucleotide appears in *vhhA*, and the upper one appears in *vhhB*. The position of the *Hin*dIII site in the *vhh* gene, starting at nucleotide 1020, is indicated by underlining.

VHR2 were also cloned into pCR2.1-TOPO. Fifteen clones containing the expected 1.4-kbp insert were obtained. The DNA from six clones was partially sequenced, and all were found to contain the *vhhB* gene. One plasmid, p645H2-1, was selected for further study. Attempts to clone the PCR products obtained with the primers VHF3 and VHR3 into pCR2.1-TOPO were unsuccessful.

Characterization of cloned hemolysin genes. The ORFs of *vhhA* and *vhhB* were both found to be 1,254 nucleotides long and are predicted to encode identical polypeptides of 418 amino acids with a deduced molecular mass of 47.3 kDa (Fig. 3). The nucleotide sequences of *vhhA* and *vhhB* are 98.8% identical and differ at only 15 nucleotide positions. The G+C content of both genes was 46%.

By comparing the nucleotide sequences of the cloned DNA contained in pVHHH1 and pVH2H with those of the *vhhA* and *vhhB* genes, it was found that both the *Hin*dIII fragments in the two plasmids carried a *vhhA* gene fragment (data not shown).

Analysis of the deduced amino acid sequence of VHH for homology to other sequences in the GenBank database revealed a high degree of identity (85.6%) to the TL hemolysin of *V. parahaemolyticus* (35) (Fig. 4). The VHH hemolysin also showed strong homology to the lecithinases of *V. mimicus* (65.3% identity) (11) and *V. cholerae* (64.3% identity). Three cysteine residues, at positions 49, 357, and 378 for VHH, were located at the same relative position in the four proteins.

The *vhh* gene confers hemolytic activity on *E. coli*. The plasmids p645H2-1 and p645H15-1, containing the ORFs of *vhhA* and *vhhB* respectively, each conferred weak hemolytic activity on *E. coli* (as determined on rainbow trout blood agar) (Fig. 5B and C). This was somewhat surprising given that all *vhh* promoter sequences were missing from both plasmids. The hemolytic activity conferred by p645H2-1, which contains the *vhhB* gene with 61 nucleotides of the promoter immediately upstream of the translational start codon, was substantially higher, confirming that the *vhh* gene is functional in *E. coli* (Fig. 5D). The zones of hemolysis were opalescent and reddish in color, resembling those of *V. harveyi* when grown on the same agar.

Expression of *vhh* genes in *V. harveyi*. RNA transcripts from the *vhh* genes in strains VIB 571, VIB 645, VIB 646, and VIB 648 were assayed by Northern blot hybridization. Whereas VIB 648 produced a strong hybridization signal, no detectable RNA was found in extracts prepared from VIB 571, VIB 645, and VIB 646 (data not shown).

DISCUSSION

V. harveyi hemolysin has been regarded as a putative pathogenicity factor in salmonids (40). Therefore, it was considered important to identify the genetic basis for hemolysin production by *V. harveyi*. Initially four pairs of degenerate primers, designed to anneal to conserved sequences in 10 hemolysin

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VML  MNRIIGDFIRKVDLTSNLSNDRALLIDETNVSFTIQERSDQICSVYFQPNKKKTMKTRLS 60
VCL  ------------------------------------------------------MKKRLS 6
VHH  ------------------------------------------------------MNKTIT 6
VPTL ------------------------------------------------------MMKKTIT 7
                                                               *:. ::

VML  LLIAGLASLSATAATEPWA--EAQPVTRAQVEQAQGKQTYTYVRCWYRPAATHDDPYTTW 118
VCL  ILIAGLASLSVNAATEPWASPEAEVLSRAQIQQVQGKQTYTYVRCWYRPAATHDDPYTTW 66
VHH  LLSALLLPLSFAHAAEPTLS--PEMVSASQVRSAQAKQTYTYVRCWYRTSYSKDEPATDW 64
VPTL LLTA-LLPLASAVAEEPTLS--PEMVSASEVISTQENQTYTYVRCWYRTSYSKDDPATDW 64
     :* * * .*:   * **     .: :: ::: ..* :***********.: ::*:* * *

VML  EWAKNADGSDYTINGYWWSSISHKNMFYTDAKPDAIKERCNETLGVTHETADITYFAADT 178
VCL  EWAKNADGSYYTIQGYWWSSIRQKNMFYTTVQPETLLERCEETLGVNHDFADITYFAADH 126
VHH  EWAENPDGSYFTLDGYWWSSVSFKNMFYTDTPQSVIKQRCEQTLDLANENADITFFAADN 124
VPTL EWAKNEDGSYFTIDGYWWSSVSFKNMFYTNTSQNVIRQRCEATLDLANENADITFFAADN 124
     ***:* *** :*::******:  ****** .  ..: :**: **.: :: ****:****

VML  RASYNHTIWNNDSAAQPNKINKVIVFGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFV 238
VCL  RFSYNHTIWSNDPEVQSNRISKVIAFGDSLSDTGNIFNASQWRFPNPDSWFLGHFSNGFV 186
VHH  RFSYNHTIWSNDPVMQPDQINKVVALGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFV 184
VPTL RFSYNHTIWSNDAAMQPDQINKVVALGDSLSDTGNIFNASQWRFPNPNSWFLGHFSNGFV 184
     * *******.**.  *.::*.**:.:********************:************

VML  WTEYLAQGLNVPIYNWAVGGAAGRNQYVALTGVYEQVSSYLTYMQLAKNYQPENSLFTLE 298
VCL  WTEYLAQGLNVPLYNWAVGGAAGRNQYVALTGVYEQVSSYLSYIALAKNYQPENSLFTLE 246
VHH  WTEYIAQAKNLPLYNWAVGGAAGENQYIALTGVGEQVSSYLAYAKLAKNYKPANTLFTLE 244
VPTL WTEYIAKAKNLPLYNWAVGGAAGENQYIALTGVGDQVSSYLTYAKLAKNYKPANTLFTLE 244
     ****:*:. *:*:**********.***:***** :******:*  *****:* *:*****

VML  FGLNDFMNYNRSLAEVKADYSSALIRLVDARAKNIVLLTLPDATRAPQFQYSTQEQIETV 358
VCL  FGLNDFMNYNRSLADVKADYSSALIRLIDAGAKNLVLMTLPDATRAPQFQYATQEQIDTV 306
VHH  FGLNDFMNYNRSVPEVKSDYAEALIKLTDAGAKNLLLMTLPDATRAPQFTYSTQEEINKI 304
VPTL FGLNDFMNYNRGVPEVKADYAEALIRLTDAGAKNFMLMTLPDATKAPQFKYSTQEEIDKI 304
     **********.:.:**:**:.***:* ** ***::*:******:**** *:***:*:.:

VML  RSKIIGMNAFIREQARYYQMQGIRIALFDAHALFDSMTANPEQHGFANASSPCLDIQRSS 418
VCL  RSKIIGMNAFIREQARYYQMQGIRIVLFDAYTLFDSITTQPEQHGFANANSPCLDIRRNS 366
VHH  RAKIVEMNEFIKAQAAYYTAQGYNVTLYDTHALFESLTANPEQHGFVNASQACQDINRSS 364
VPTL RAKVLEMNEFIKAQAMYYKAQGYNITLFDTHALFETLTSAPEEHGFVNASDPCLDINRSS 364
     *:*:: ** **: ** **  ** .:.*:*:::**:::*: **:***.**...* **.*.*

VML  AADYLYSHSLSAECAAQGSDRFVFWEVTHPTTATHRYIAQHILAT--EMGQFPL 470
VCL  AADYLLSHSLSAECAKQGSDRFVFWEVTHPTTAIHHYLAEQILAT--EMAQFPL 418
VHH  SVDYLYHHSLRSECASSGSDKFVFWDVTHPTTATHHYVAEKMLESTNQLSNHPF 418
VPTL SVDYMYTHALRSECAASGAEKFVFWNVTHPTTATHRYVAEKMLESSNNLAEYRF 418
     :.**:  *:* :*** .*:::****:******* *:*:*:::* :  ::.:. :
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FIG. 4. Alignment of the deduced amino acid sequence of the *V. harveyi* VIB 645 VHH hemolysin with those of related proteins. VHH, hemolysin from *V. harveyi* VIB 645; VPTL, TL hemolysin from *V. parahaemolyticus* (35) (GenBank accession no. M36437); VML, lecithinase from *V. mimicus* (11) (GenBank accession no. AF035162); VCL, lecithinase from *V. cholerae* (GenBank accession no. U50074). Symbols: *, identical residues in all sequences; :, conserved substitutions; ., semiconserved substitutions; -, gaps introduced during the alignment process. Numbers on the right refer to the amino acid residue at the end of each line.

genes from *Vibrio* and *Aeromonas* species, were used in an attempt to amplify DNA from *V. harveyi* by PCR, but no products were obtained (data not shown). Instead, probes specific for the *V. mimicus vmhA* hemolysin gene (12) and the *V. parahaemolyticus tl* hemolysin gene (35), were used to analyze DNA from *V. harveyi* by Southern blotting and hybridization. The *vmhA* gene was selected because it showed strong homology with six other hemolysin genes (data not shown), whereas the *tl* gene of *V. parahaemolyticus* was selected because this species is the most closely related to *V. harveyi* (25). Whereas the VPHP probe (produced by PCR amplification of the *tl* gene of *V. parahaemolyticus*) showed strong hybridization to the digests of total DNA from all of the *V. harveyi* isolates examined (Fig. 1), the *V. mimicus vmhA* probe (VMHP) did not hybridize to the DNA of any *V. harveyi* isolate (data not shown). This suggested that *V. harveyi* contains a hemolysin gene with strong similarity to the *tl* gene of *V. parahaemolyticus*. Therefore, the *V. parahaemolyticus* probe, VPHP, was employed for the initial cloning of the hemolysin gene from *V. harveyi*.

An interesting finding was the presence of two hemolysin gene copies in the most pathogenic strain, *V. harveyi* VIB 645, and only one copy in the majority of other *V. harveyi* strains. Strain VIB 648 also appeared to contain two gene copies, but these were arranged in a different manner compared to VIB 645, as judged from Southern blotting and hybridization analysis (Fig. 1). Both gene copies from VIB 645 (designated *vhhA* and *vhhB*) were individually cloned into *E. coli*. The nucleotide

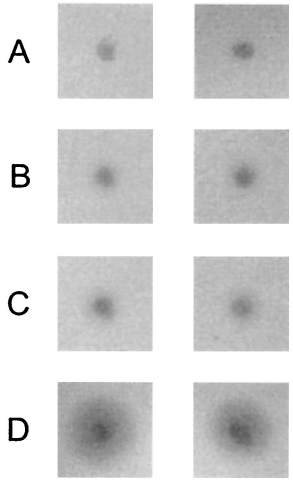

FIG. 5. Expression of the *vhh* gene of *V. harveyi* in *E. coli*. Duplicate rainbow trout blood agar plates were inoculated as described in Materials and Methods. A. *E. coli* TOP10F′; B, *E. coli* TOP10F′ containing p645H1-1 (*vhhA*); C, *E. coli* TOP10F′ containing p645H1-15 (*vhhB*); D, *E. coli* TOP10F′ containing p645H2-1 (*vhhA* including 61 nucleotides of promoter DNA upstream of the translational start codon).

sequences of *vhhA* and *vhhB* were not identical but were very similar (98.8% identity), suggesting that the duplication of the *vhh* gene was a relatively recent event. The predicted amino acid sequences encoded by *vhhA* and *vhhB* were identical. The plasmid p645H2-1, which contained the ORF of *vhhB* together with 61 nucleotides of the promoter immediately upstream of the translational start codon, expressed high levels of hemolysin in *E. coli*, confirming that the *vhhA* gene was functional. We do not know at this stage if the two *vhh* genes contribute equally to hemolysin activity in *V. harveyi*. Further studies on the promoter sequences of the *vhhA* and *vhhB* genes may shed some light on this point.

Northern blot analysis revealed that VIB 648, which contains two *vhh* genes, expressed a high level of *vhh*-specific RNA transcript, whereas VIB 571, VIB 645, and VIB 646 produced no detectable *vhh* RNA under the experimental conditions used here. The result for VIB 645 was unexpected and suggests that the high level of hemolytic activity displayed by this strain is not a consequence of increased *vhh* gene expression. Conversely, for VIB 648, an increase in transcription, possibly for only one of the two *vhh* genes, is likely to be a contributing factor towards the high level of hemolytic activity shown by this strain.

The duplication of the *vhh* gene in *V. harveyi* is a phenomenon similar to that previously described for the hemolysin (*hly*) determinant of *E. coli* (13, 14) and the duplicated *tdh* genes of *V. parahaemolyticus* (23). However, unlike the *tdh* gene and the *hly* determinant, which have a much higher A+T content than the corresponding chromosomal DNA, the *vhh* gene had an A+T content (54.1%) similar to that of the chromosomal DNA (an average of 52 to 54%) (4).

The *V. harveyi* VHH protein shows extensive homology with the *V. parahaemolyticus* TL protein, also called lecithin-dependent hemolysin, which has been shown to confer thermolabile hemolytic activity (32). Previously, we have partially purified the VHH hemolysin of *V. harveyi* VIB 645 and found that the hemolytic activity was thermolabile (unpublished data). A BLAST search revealed that the VHH protein is also highly homologous to the lecithinases of *V. mimicus* and *V. cholerae*, suggesting that VHH is a lecithinase-like protein. The positions and numbers of cysteine residues in the four proteins were identical (except for an extra cysteine in VML towards the N-terminal end of the protein), suggesting that they share similar secondary structures. Interspecies relatedness has also been reported with the thermostable direct hemolysin originally identified in *V. parahaemolyticus* (22). Sequence homology with the gene encoding this hemolysin has been found in *V. cholerae* non-O1, *V. mimicus*, and *V. hollisae* (39).

Although gene duplication is acknowledged to be a mechanism that can lead to increased levels of the encoded product, this does not appear to be the case for overnight cultures of VIB 645. Nevertheless, it remains a distinct possibility that one of the *vhh* genes is expressed in a manner different than that of the other. There may be a high, but transient, level of expression of one gene that was not detected here by Northern blot analysis. Further studies on the molecular basis of hemolysin production, which could include sequencing and characterization of the promoter regions of the *vhh* genes, will be necessary to explain the high level of hemolytic activity shown by VIB 645.

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